REMARKS

Applicants respectfully request reconsideration of the present application.

1. Disposition of the Claims and Specification

Claims 1-28 are pending. Claims 8, 10, 15 and 18-28 are withdrawn. Claims 29-30 are newly added. Claims 1-7, 9, 11-14, 16-17 and 29-30 are ready for prosecution on the merits. Claims 1-2, 5, 7, 11-13 and 17 are currently amended. Claims 1-2, 5, 11 and 17 are amended to remove non-elected subject matter. Claim 7 is amended at the suggestion of the examiner.

Support for newly added claim 29 may be found in the specification, for example, at page 23, lines 26-31. Support for newly added claim 30 may be found in the specification, for example, at page 23, lines 9-14 and at page 26, lines 1-7.

Support for the amendment to claim 7 may be found in the specification, for example, at page 6, lines 6-8. Support for the amendment to claim 1 may be found in the specification, for example, at page 6, lines 19-25, at page 19, lines 6-10 and at page 23, lines 26-31. Support for the amendment to claim 12 may be found in the specification, for example, at page 14, lines 32-35. Support for the amendment to claim 13 may be found in the specification, for example, at page 18, lines 18-23.

Applicants have amended the title and the specification at the request of the examiner. Because the foregoing amendments do not introduce new matter, entry thereof by the Examiner is respectfully requested.

2. Specification

The examiner has objected to the specification for containing embedded hyperlinks. Applicants have amended the specification to remove the embedded hyperlinks.

The examiner has further objected to the title of the invention for not being descriptive and requires a new title clearly indicative of the invention. Applicants have amended the title to read "A MEMBRANE ASSOCIATED PROTEIN, A NUCLEIC ACID MOLECULE

ENCODING THE PROTEIN, AND METHOD OF DETECTION" at the suggestion of the examiner. Applicants respectfully request withdrawal of the objections to the specification.

3. Claim Objections

Claims 1-2, 5, 11 and 17 are objected to for reciting non-elected groups of sequences. Applicants have amended claims 1-2, 5, 11 and 17 to remove non-elected groups of sequences.

Claim 7 is objected to for reciting a non-elected group, specifically a host cell and a transgenic organism. The examiner notes that the objection can be overcome by amending the claim to recite "a recombinant host cell..." or "an isolated host cell..." Applicants have amended claim 7 to recite "a recombinant host cell" as suggested by the examiner. Support for the amendment to claim 7 may be found in the specification, for example, at page 6, lines 6-8. Applicants respectfully request withdrawal of the objections to claims 1-2, 5, 7, 11 and 17.

4. Claim Rejections – 35 U.S.C. § 101

In paragraph 5 of the office action, the examiner rejected claims 1-7, 9, 11-14 and 16-17 under 35 U.S.C. § 101 because the invention is allegedly not supported by either a credible, specific or substantial asserted utility or a well established utility. The examiner notes that the specification discloses the claimed polypeptide and polynucleotide as a membrane associated protein, specifically MEMAP-28. The examiner also points out that the specification, at page 36, lines 29-30, discloses that MEMAP "appears to play a role in cell proliferative, autoimmune/inflammatory, neurological, and gastrointestinal disorders." Office Action at 6.

The examiner reasons, however, that the specification "does not teach any significance or functional characteristics of the MEMAP-28 polynucleotide or polypeptide. The specification also does not disclose any methods or working examples that indicate MEMAP-28 is involved in any activity or disorder." Office Action at 6. The examiner further points out that while the specification asserts a patentable utility in treating or

preventing "a disorder associated with decreased expression or activity of MEMAP", such utility is not specific or substantial. Office Action at 8. The examiner reasons that the specification "does not disclose diseases associated with a mutated, deleted or translocated MEMAP polynucleotide (SEQ ID NO: 65) or polypeptide (SEQ ID NO: 28)." Office Action at 8. Applicants respectfully request reconsideration and withdrawal of the rejection.

A. The specification teaches the significance and functional characteristics of MEMAP-28 in cell proliferative, immune and inflammatory disorders

As the examiner has already pointed out, page 1, lines 4-6 and page 23, lines 33-35 state that the proteins and sequences of the invention are useful in the diagnosis, treatment and prevention of cell proliferative disorders, immune disorders, and inflammatory disorders. Specifically, page 36, lines 34-36 of the specification states that the claimed protein and nucleotide sequence "may be administered to treat or prevent a disorder associated with decreased expression or activity of MEMAP" and the specification, on page 37, goes on to provide examples of such disorders. Similarly, page 38, lines 29-32 explains that the claimed invention may also be used to treat or prevent a disorder associated with "increased expression or activity of MEMAP." More particularly, the specification, at page 37, lines 1-8, specifically discloses the use of the claimed invention in the treatment and/or prevention of cancer.

B. Post-filing publications confirm the utility of MEMAP-28 in cell proliferative, immune and inflammatory disorders

According to the results of the attached sequence alignment, performed with SEQ ID NO: 28 of the instant invention, the protein identified in the specification having an amino acid sequence corresponding to SEQ ID NO: 28 is 100% identical to BPIL1. *See* Alignment (Exhibit 1). The annotation of BPIL1 (Exhibit 2) and associated post-filing article by Mulero *et al.*, IMMUNOGENETICS 54:293-300 (2002) (Exhibit 3), show that BPIL1 is a member of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family and is related to the bactericidal/permeability-increasing protein (BPI).

Mulero *et al.* describes BPIL1 as being downregulated in larynx carcinoma tissue. *See* Mulero *et al.* at 296. The authors further explain that BPIL1 is overexpressed in inflamed disease tissues, which suggests "that these genes may play a role in innate immunological functions ... in inflammation, host defense or pain." *See* Mulero *et al.* at 299. Because the invention has at least one substantial and credible utility as set forth in the specification and confirmed by another publication, i.e. the treatment and/or prevention of larynx carcinoma, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 101.

5. Claim Rejections – 35 U.S.C. § 112, first paragraph

In paragraph 6 of the office action, the examiner rejected claims 1-7, 9, 11-14 and 16-17 under 35 U.S.C. § 112, first paragraph, because the claimed invention is allegedly not supported by either a specific and substantial asserted utility or a well established utility. The examiner reasons that one skilled in the art would not know how to use the claimed invention.

Applicants respectfully disagree with the examiner. Applicants have established a specific and substantial asserted utility, as described above in Section 5. Therefore, Applicants respectfully request withdrawal of this rejection.

6. Claim Rejections – 35 U.S.C. § 112, first paragraph – enablement of claims 1-7, 9, 11-14 and 16-17

In paragraph 6a of the office action, the examiner rejected claims 1-7, 9, 11-14 and 16-17 under 35 U.S.C. § 112, first paragraph, for allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The examiner reasons that the specification "does not enable the claimed naturally-occurring, biologically active, or immunogenic fragments and variants of MEMAP." Office Action at 11.

First, the examiner reasons that the specification "does not disclose methods or examples to enable one skilled in the art to obtain a 'natural' MEMAP." Office Action at 11. Applicants respectfully disagree with the examiner. However, to expedite prosecution, Applicants have amended claims 1 and 11 to delete the reference to "naturally occurring"

variants of MEMAP. Support for the amendment to claims 1 and 11 may be found in the specification, for example at page 23, lines 9-14 and lines 26-31. Applicants respectfully request reconsideration and withdrawal of the rejection.

Second, the examiner states that the specification does not disclose "methods or working examples that show how to use 'biologically active' fragments or that describe the specific activity associated with the fragments." Office Action at 11. Applicants respectfully disagree with the examiner. For example, Table 2 of the instant specification discloses potential phosphorylation sites, potential glycosylation sites, a signal peptide domain, a transmembrane domain, membrane glycoprotein signature sequences, and an olfactory ligand binding domain of SEQ ID NO: 28. Applicants submit that one of ordinary skill in the art would know how to make and use biologically active fragments of SEQ ID NO: 28 by referring to Table 2 of the specification. Applicants respectfully request reconsideration and withdrawal of the rejection.

Third, the examiner believes that "an 'immunogenic fragment' of the amino acid sequence of SEQ ID NO: 28 gives rise to an antibody that is not specific for SEQ ID NO: 28." Office Action at 11. The examiner explains that such an immunogenic fragment elicits a general immune response and that the specification fails to teach one skilled in the art how to use non-specific antibodies. Applicants respectfully disagree with the examiner. Applicants note that they are not claiming any antibodies, whether specific or non-specific to SEQ ID NO: 28. Rather, claim 1 merely recites an immunogenic fragment. Therefore, the specification need not teach of one skill in the art how to use non-specific antibodies. Applicants respectfully request reconsideration and withdrawal of the rejection.

Fourth, the examiner reasons that while the specification discloses "variants" of the particular polynucleotide and polypeptide sequence, the specification does not teach: (a) an amino acid sequence "with at least 70% sequence identity to ... SEQ ID NO: 28"; (b) a polynucleotide sequence "having at least 90% sequence identity to ... SEQ ID NO: 65"; or (c) "any polynucleotide comprising at least 60 contiguous nucleotides of SEQ ID NO: 65." Office Action at 12. Further, the examiner believes that "the specification does not teach

functional or structural characteristics of the polynucleotides or polypeptides in the context of a cell or organism." Office Action at 12. The examiner refers to problems of predicting protein and DNA structure from sequence data, and states that "certain positions in the sequence are critical to the protein's structure/function relationship." Office Action at 12.

Applicants respectfully disagree with the examiner. Applicants submit that one of skill in the art would be able to make a polypeptide and polynucleotide with a certain level of identity, i.e., 90% and/or 95% identity, to a reference sequence. Further, one of skill in the art would also be able to make a fragment having 60 contiguous nucleotides of SEQ ID NO: 65. In this regard, Applicants point to Table 2 of the instant specification. Table 2 discloses various structural characteristics of the claimed polypeptide including: (1) potential phosphorylation sites (T408, T98, S126, S170, T334); (2) potential glycosylation sites (N96, N151, N293, N332); and (3) signature sequences, motifs and domains such as signal peptides (M1-A20), transmembrane domains (L10-N30), membrane glycoprotein signature (L9-V101 and L64-Q457), and an olfactory ligand binding domain (T67-S452). Applicants respectfully request reconsideration and withdrawal of the rejection.

7. **Claim Rejections – 35 U.S.C. § 112, first paragraph – enablement of claims 16-17**

In paragraph 6b of the office action, the examiner has rejected claims 16-17 because the claims allegedly recite "an intended use of the MEMAP polypeptide for treatment or administration in an animal." Office Action at 13. The examiner reasons that the specification "does not teach how to use a MEMAP polypeptide without undue experimentation for the treatment of a disease in an animal." Office Action at 13. The examiner further states that the rejection can be overcome by deleting the phrase "pharmaceutically acceptable excipient."

Applicants respectfully disagree with the examiner. Applicants believe that one of ordinary skill in the art would be able to make and use the particular invention as recited in claims 16-17, without undue experimentation. Applicants claim a "composition comprising an effective amount of a polypeptide of claim 1 and a pharmaceutically acceptable excipient", which is supported in the specification at page 7, lines 21-28 and at page 47, lines 23-25.

Applicants note that the claims 16 and 17 do not recite a method of use, as suggested by the examiner. As such, Applicants respectfully request reconsideration and withdrawal of the rejection.

8. Claim Rejections – 35 U.S.C. § 112, first paragraph – written description

The examiner has rejected claims 1-7, 9, 11-14 and 16-17 under 35 U.S.C. § 112, first paragraph. The examiner reasons that the specification fails to disclose any functional or structural characteristics of the claimed sequences. The examiner also asserts that the description of a single polynucleotide sequence and polypeptide sequence "is not adequate written description of an entire genus of functionally equivalent polynucleotides and polypeptides which incorporate all variants and fragments with at least 70% sequence identity to ... SEQ ID NO: 28, at least 90% sequence identity to ... SEQ ID NO: 65, or at least 60 contiguous nucleotides of ... SEQ ID NO: 65." Office Action at 15.

Applicants respectfully disagree with the examiner. Applicants point to Table 2, which discloses various structural characteristics of the claimed invention. Table 2 discloses structural characteristics of the claimed polypeptide including: (1) potential phosphorylation sites (T408, T98, S126, S170, T334); (2) potential glycosylation sites (N96, N151, N293, N332); and (3) signature sequences, motifs and domains such as signal peptides (M1-A20), transmembrane domains (L10-N30), membrane glycoprotein signature (L9-V101 and L64-Q457), and an olfactory ligand binding domain (T67-S452). Applicants respectfully request reconsideration and withdrawal of the rejection.

With respect to the claimed polypeptides that are 70% identical to SEQ ID NO: 28, Applicants respectfully disagree that the specification does not provide adequate description of the entire genus of claimed sequences. However, to expedite prosecution, Applicants have amended claim 1 to recite that the claimed polypeptide variants are 90% identical to SEQ ID NO: 28. Support for the amendment to claim 1 may be found in the specification, for example, at page 23, lines 26-31.

Applicants further assert that one of ordinary skill in the art would be able to create functionally equivalent 90% identical polypeptide and polynucleotide variants by following the teachings of the specification. Specifically, by using the information from Table 2, one of ordinary skill would know to retain those portions of the sequence identified in Table 2 when creating a variant 90% identical to SEQ ID NOs: 28 and 65.

With respect claim 12, Applicants believe that the specification does provide adequate written description for a polynucleotide comprising at least 60 contiguous nucleotides of SEQ ID NO: 65. Applicants note that they have amended claim 12, as discussed below in Section 10, to recite a polynucleotide comprising at least 150 contiguous nucleotides of SEQ ID NO: 65. Support for the amendment to claim 12 may be found in the specification, for example, at page 14, lines 32-35. As such, Applicants respectfully request reconsideration and withdrawal of the rejection.

9. Claim Rejections – 35 U.S.C. § 112, second paragraph

The examiner has rejected claims 13-14 under 35 U.S.C. § 112, second paragraph. The examiner reasons that the term "specifically hybridizes" in claim 13 is a relative term, which renders the claim indefinite. The examiner explains that in "the absence of a recitation of clear hybridization conditions (e.g., 'hybridizes at wash conditions consisting of A X SSC and B % SDS at C° C'), claims 13-14 fail to define the metes and bounds" recited in the claims. Office Action at 17.

Applicants respectfully disagree with the examiner. However, to expedite prosecution, Applicants have amended claim 13 to recite clear and specific hybridization conditions. Support for the amendment to claim 13 may be found in the specification, for example, at page 18, lines 18-23. Applicants respectfully request reconsideration and withdrawal of the rejection.

10. Claim Rejections – 35 U.S.C. § 102

Claim 12 is rejected under 35 U.S.C. § 102(a) as being anticipated by Genbank Accession No. AI834221. The examiner reasons that the sequence disclosed in the cited

Genbank accession teaches an isolated polynucleotide comprising at least 60 contiguous nucleotides of SEQ ID NO: 65. Specifically, the cited sequence comprises 110 contiguous nucleotides of SEQ ID NO: 65.

Applicants have amended claim 12 to recite "at least 150 contiguous nucleotides" of SEQ ID NO: 65. Support for the amendment to claim 12 may be found in the specification, for example, at page 14, lines 32-35. Applicants respectfully request withdrawal of the rejection.

11. Conclusion

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 10/7/04

By _____

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3393430CD1_PRT_28_PF-0731-USN 458 aa

21667210 458 aa

BKL Data for gene:Member of the LBP-BPI-CETP family, contains one LBP-BPI-CETP C-terminal domain, has weak similarity to human BPI, which binds to gram-negative bacteria and is associated with acute pancreatitis, enteritis and cirrhosis

Match: Length=458, Identity: 100%, Similarity:100%, Query Overlap: 100%, Subject Overlap: 100%, E-value:0.0, Score:877

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Query: 1   MAWASRLGLLLALLLPVVGASTPGTVVRLNKAALSYVSEIGKAPLQRALQVTVPHFLDWS 60
           MAWASRLGLLLALLLPVVGASTPGTVVRLNKAALSYVSEIGKAPLQRALQVTVPHFLDWS
Sbjct: 1   MAWASRLGLLLALLLPVVGASTPGTVVRLNKAALSYVSEIGKAPLQRALQVTVPHFLDWS 60

Query: 61  GEALQPTRIRILNVHVPRLHLKFIAGFGVRLLAAANFTFKVFRAPEPLELTLPVELLADT 120
           GEALQPTRIRILNVHVPRLHLKFIAGFGVRLLAAANFTFKVFRAPEPLELTLPVELLADT
Sbjct: 61  GEALQPTRIRILNVHVPRLHLKFIAGFGVRLLAAANFTFKVFRAPEPLELTLPVELLADT 120

Query: 121 RVTQSSIRTPVVSISACSLFSGHANEFDGSNSTSHALLVLVQKHIKAVLSNKLCLSISNL 180
           RVTQSSIRTPVVSISACSLFSGHANEFDGSNSTSHALLVLVQKHIKAVLSNKLCLSISNL
Sbjct: 121 RVTQSSIRTPVVSISACSLFSGHANEFDGSNSTSHALLVLVQKHIKAVLSNKLCLSISNL 180

Query: 181 VQGVNVHLGTLIGLNPVGPESQIRYSMVSVPTVTSDYISLEVNAVLFLLGKPIILPTDAT 240
           VQGVNVHLGTLIGLNPVGPESQIRYSMVSVPTVTSDYISLEVNAVLFLLGKPIILPTDAT
Sbjct: 181 VQGVNVHLGTLIGLNPVGPESQIRYSMVSVPTVTSDYISLEVNAVLFLLGKPIILPTDAT 240

Query: 241 PFVLPRHVGTEGSMATVGLSQQLFDSALLLLQKAGALNLDITGQLRSDDNLLNTSALGRL 300
           PFVLPRHVGTEGSMATVGLSQQLFDSALLLLQKAGALNLDITGQLRSDDNLLNTSALGRL
Sbjct: 241 PFVLPRHVGTEGSMATVGLSQQLFDSALLLLQKAGALNLDITGQLRSDDNLLNTSALGRL 300

Query: 301 IPEVARQFPEPMPVVLKVRLGATPVAMLHTNNATLRLQPFVEVLATASNSAFQSLFSLDV 360
           IPEVARQFPEPMPVVLKVRLGATPVAMLHTNNATLRLQPFVEVLATASNSAFQSLFSLDV
Sbjct: 301 IPEVARQFPEPMPVVLKVRLGATPVAMLHTNNATLRLQPFVEVLATASNSAFQSLFSLDV 360

Query: 361 VVNLRLQLSVSKVKLQGTTSVLGDVQLTVASSNVGFIDTDQVRTLMGTVFEKPLLDHLNA 420
           VVNLRLQLSVSKVKLQGTTSVLGDVQLTVASSNVGFIDTDQVRTLMGTVFEKPLLDHLNA
Sbjct: 361 VVNLRLQLSVSKVKLQGTTSVLGDVQLTVASSNVGFIDTDQVRTLMGTVFEKPLLDHLNA 420

Query: 421 LLAMGIALPGVVNLHYVAPEIFVYEGYVVISSGLFYQS 458
           LLAMGIALPGVVNLHYVAPEIFVYEGYVVISSGLFYQS
Sbjct: 421 LLAMGIALPGVVNLHYVAPEIFVYEGYVVISSGLFYQS 458
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Schematic Colors:

Very Strong	Strong	High	Moderate	Low	Weak
>95%	80-95%	45-80%	35-45%	25-35%	20-25%

Quick Search: [ ] Name/ID

Proteome
BioKnowledge® Library
IPPD ™
Incyte patented

3393430CB1_DNA_65_PF-0731-USN

Protein containing a lipopolysaccharide-binding protein, bactericidal permeability-increasing protein, or cholesteryl ester transfer protein C-terminal domain, has weak similarity to bactericidal permeability-increasing protein (human BPI)

Gene Symbol/Synonyms	3393430CB1_DNA_65_PF-0731-USN	
Corresponding Human Orthologs	BPIL1 INCY:961033.FL1	Tools
Gene Families	Secreted	
Gene Ontology		
Molecular Function	Phospholipid binding [P]; Lipopolysaccharide binding [P] [details]	BIOKNOWLEDGE RETRIEVER
Biological Process	Cellular defense response [P]; Defense response [P]; Acute-phase response [P]; Response to pathogenic bacteria [P] [details]	
Cellular Component	Extracellular space [P] [details]	
Expression		
Organ/Tissue	Salivary gland [E]; Tonsil [E]; Trachea [E] [details]	
Cell Type		
Tumor Type		
Disease		
Diagnostic Marker		
Therapeutic Target		
Molecular Mechanism		
Negative Correlation		
Sequence		
Full	mawasrl...sglfyqs (1..458; 458 aa) pI: 8.91 MW: 49174 TM: 1 [P]	PROTEIN
Gene	Chromosome: 20q11.22 Introns:	

3D Structure (PDB) 1BP1_ (22%); 1EWF_A (22%) [details] SEQUENCE
Domain LBP / BPI / CETP family, N-terminal domain... [details]

Related Proteins
H. sapiens BPIL1 (100%); INCY:1100723 (26%)... [details]
Patents 7479161CB1_DNA_20_PI-0346-PCT (28%)... [details]
M. musculus 2310034L21RIK (68%); RYA3 (26%)... [details]
R. norvegicus
D. rerio
D. melanogaster
C. elegans F55B12.5 (26%) [details]
S. pombe
S. cerevisiae
Fungal Pathogens
C. albicans
Others

BLAST SUMMARY

LifeSeq® Foundation Release 13
Human Transcripts INCY:961033 Incyte Gene

Transcript ID		Description
INCY:961033.FL1	[BPIL1]	458-aa form
INCY:961033.FL2		440-aa splice form, lacks LBP_BPI_CETP_C Pfam domain

Interactions
Protein-Protein
Complexes

Gene Regulation
Induced by
Repressed by
Not Affected by

Protein Modifications

GenBank #	**PIR #**	**SWISS-PROT # Q8N4F0**
Locus Link # 80341	**Unigene # 257045**	**OMIM #**

Name

- DJ726C3.2 "hypothetical protein dJ726C3.2"

References

1079294. Bingle, C. D., and Bingle, L. Characterisation of the human plunc gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern Biochim Biophys Acta 1493, 363-7 (2000).

1195746. Mulero, J. J., Boyle, B. J., Bradley, S., Bright, J. M., Nelken, S. T., Ho, T. T., Mize, N. K., Childs, J. D., Ballinger, D. G., Ford, J. E., and Rupp, F. Three new human members of the lipid transfer/lipopolysaccharide binding

protein family (LT/LBP). Immunogenetics 54, 293-300. (2002).

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ORIGINAL PAPER

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Three new human members of the lipid transfer/lipopolysaccharide binding protein family (LT/LBP)

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Abstract We have identified three novel, rarely expressed human genes that encode new members of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family based on sequence homology. BPI and other members of the LT/LBP family are structurally related proteins capable of binding phospholipids and lipopolysaccharides. Real-time PCR studies indicate that *BPIL1* and *BPIL3* are highly expressed in hypertrophic tonsils. In situ hybridization analysis of *BPIL2* shows prominent expression in skin specimens from psoriasis patients. *BPIL1* and *BPIL3* map to Chromosome 20q11; thus, these novel genes form a cluster with *BPI* and two other members of the LT/LBP gene family on the long arm of human Chr 20. *BPIL2* maps to Chr 22q13. The exon/intron organization of all three genes is highly conserved with that of *BPI*, suggesting evolution from a common ancestor.

Sequence data for this article have been deposited with the GenBank data library under accession numbers AF465765 (*BPIL1*), AF465766 (*BPIL2*) and AF465767 (*BPIL3*)

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Keywords Psoriasis · Tonsil · Lipoamino acids · Human Chromosome 20 · BPI

Introduction

The current members of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family are bactericidal/permeability-increasing protein (*BPI*), cholesterol ester transfer protein (*CETP*), phospholipid transfer protein (*PLTP*) and lipopolysaccharide binding protein (*LBP*). These molecules contain lipid-binding sites and appear to have a common ability to bind phospholipids and lipopolysaccharide (LPS) (Hailman et al. 1996; Bruce et al. 1998a), albeit with different affinities. These proteins share significant amino acid similarity and exon/intron organization suggesting that they arise from a common ancestor (Tu et al. 1995; Hubacek et al. 1997).

CETP and PLTP are human plasma proteins and both play an important role in the catabolism of high-density lipoproteins (HDL), thus influencing the pathogenesis of atherosclerosis (Bruce et al. 1998b; Huuskonen et al. 2001). CETP is an important determinant of lipoprotein composition because of its capacity to mediate the net transfer of triglycerides and cholesteryl esters between lipoprotein particles such as HDL. PLTP mediates the exchange and transfer of different phospholipids between HDL particles.

LBP is a plasma protein, while BPI is found in specific granules of neutrophils and eosinophils (Calafat et al. 1998); both proteins bind bacterial endotoxins and modulate the host response to Gram-negative bacterial infections (Schumann et al. 1990; Elsbach and Weiss 1998). LBP promotes endotoxin-mediated cell activation via an interaction with the cell surface protein CD14 (Wright et al. 1990). BPI is bactericidal (Weiss et al. 1978), neutralizes endotoxin (Marra et al. 1990), and may have therapeutic value against sepsis (Lin et al. 1996). The crystal structure of the human BPI protein has been solved (Beamer et al. 1997) and consists of a boomerang-shaped molecule composed of two domains that share a

very similar fold. Each domain includes a hydrophobic pocket on the concave surface of the boomerang. The hydrophobic pockets bind a phospholipid molecule through interactions with the acyl chains of the lipid.

We report here the cloning, characterization and mapping of three previously unknown members of the LT/LBP protein family identified by amino acid similarity. These genes have been designated as bactericidal/permeability-increasing protein-like 1, 2 and 3 (*BPIL1*, *BPIL2* and *BPIL3*) (http://www.gene.ucl.ac.uk/nomenclature/).

Materials and methods

cDNA isolation

BPIL1 and *BPIL3* were identified from a human trachea cDNA library using the screening by hybridization (SBH) approach (Drmanac and Drmanac 1999). Their full-length sequence was obtained by rapid amplification of 5'-cDNA ends (RACE) (Mutimer et al. 1998) from a trachea cDNA library for *BPIL1* and from a tonsil cDNA library for *BPIL3*. *BPIL2* was also identified by the SBH approach, as a rarely expressed transcript in a fetal skin cDNA library. Full-length sequence was obtained by RACE using the same cDNA library.

Real-time PCR

RNA from hypertrophic and normal tonsils (Biochain Institute) was reverse transcribed to first-strand cDNA using MultiScribe Reverse Transcriptase (PE Biosystems) as described by the manufacturer. Two reactions were performed on each mRNA sample, either in the presence or absence of reverse transcriptase. Each reaction contained 1 μg of poly(A)$^+$ RNA in a 50-μl total volume. The thermal cycling parameters for the reactions were 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. Amplification of the cDNA was performed in the presence of SYBR Green dye using a GeneAmp 5700 Sequence detection system (PE Biosystems) with gene-specific primers, designed using Primer Express (PE Biosystems). Forward and reverse primer sequences were designed for *BPIL1* (5'-TGTCCACCTGGGCACCTTAA-3' and 5'-GGAATAGCGGATCTGGGACTC-3') and for *BPIL3* (5'-CCAACCTCAGTGACCCCTG-3' and 5'-TGGTGCGGACATCAGAACATA-3'). The quantitative PCR was carried out using a SYBR Green PCR master mix (PE Biosystems) in a final reaction volume of 25 μl, containing 1 μl of reverse-transcribed RNA product, and 300 nM concentration of the forward and reverse primers. The reaction conditions consisted of an initial denaturation step for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All cDNA quantitations were normalized to cyclophilin 40 (5'-GGGAGCGAGTTGGTCGAATT-3' and 5'-CAGTCGTGTGTCCAATGCCTT-3') (Drwinga et al. 1993) using a relative quantitation formula, as shown in the SYBR Green protocol (PE Biosystems).

Chromosomal localization of the *BPIL* genes

To determine the chromosomal localization of *BPIL1*, gene-specific PCR primers (5'-GATATCCAGTGGACTCTTCTACC-3' and 5'-CAGGTCTATACAGCCTGGAGG-3') that resulted in a 315-nt product were screened against the NIGMS human/rodent somatic cell hybrid mapping panel no. 2 (Stewart et al. 1997) and the Stanford G3 Human/Hamster Radiation Hybrid panel (Thompson et al. 1994). Gene-specific primers for *BPIL2* (5'-TCCTTTTGTGCTCCCAGAACG-3' and 5'-TAGGGCTGCTCAACCGGTAAG-3') resulted in a 406-bp band. Gene-specific primers for *BPIL3* (5'-GGATGAGAGTCATATCCTGGA-3' and 5-TGCCTGTGGACACACATTGGAAGA-3') resulted in a 1.32-kb specific band.

PCR amplification was performed using the following conditions: an initial denaturation at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by an extension of 10 min at 72 °C. All products were separated by 3% agarose-gel electrophoresis and visualized by staining with ethidium bromide. Linkage analysis, and subsequent chromosomal localization were obtained through use of the Stanford Human Genome Center RH server (http://www-shgc.stanford.edu/RH/).

Expression information

First strand human cDNA libraries from multiple tissues were screened with gene specific primers. The commercial panels (Clontech) screened were: panel I (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas), panel II (Spleen, thymus, prostate, testis, ovary, small intestine and colon), immune panel (spleen, lymph node, thymus, tonsil, bone marrow, fetal liver, peripheral blood leukocyte) and a blood fraction panel (mononuclear, resting CD8$^+$, resting CD4$^+$, resting CD14$^+$, resting CD19$^+$, activated mononuclear cells, activated CD4$^+$ and activated CD8$^+$). The panels were screened by PCR amplification using primers specific for each gene: *BPI* (5'-ACGCCAATATCAAGATCAGCG-3' and 5'-ATTCTCGAGTCATATTTTGGTCATTACTGGCAGAG-3'), *BPIL1* (5'-GATATCCAGTGGACTCTTCTACC-3' and 5'-CAGGTCTATACAGCCTGGAGG-3'), *BPIL3* (5'-CAAGCTTGCTGATGCCGGGGAGGC-3' and 5'-CATGCACTGAGTACTGGACCTTCAG-3'), *BPIL2* (5'-CATTATTGCAAGTGAAGTCAAAGC-3' and 5'-ACCATGAAGGGCTGGGACAAGATG-3') and *G3PDH* (5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3'). PCR was performed for 30 cycles using the following conditions: an initial denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 54 °C and 1 min at 72 °C, followed by an extension of 10 min at 72 °C. The amplification product for each gene was detected by analysis on agarose gels stained with ethidium bromide.

Structure modeling

Three-dimensional structural models of the BPIL proteins were generated with the GeneAtlas software package (Accelrys, San Diego, Calif.). These models were predicted based on a search of 4250 non-redundant Protein Data Bank structures (http://www.rcsb.org/pdb), using a PSI-BLAST multiple alignment sequence profile-based searching method (Myers and Miller 1988) and High Throughput homology modeling, an automated sequence and structure searching procedure (Sali and Overington 1994). The known crystal structure of BPI (Beamer et al. 1997) was the best fit structure of 4250, and was used as a template for structural overlays using Profiles-3D, a threading program that measures the compatibility of the protein model with its sequence using a 3-D profile. Using defined parameters, Profiles-3D computes a score for the model normalized by the length of the amino acid sequence.

In situ hybridization

All tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, and cut into 4-μm thick sections. Sections were placed onto Ventana's ChemMate Capillary Gap Slides (POP075). Tissues were hybridized with a DIG-labeled (Roche Molecular Biochemicals) *BPIL2*-specific antisense riboprobe. The *BPIL2* probe was derived from a 414-nt coding sequence between the primers 5'-CATTATTGCAAGTGAAGTCAAAGC-3' and 5'-ACCATGAAGGGCTGGGACAAGATG-3'. QualTek Molecular labs (Santa Barbara, Calif.) performed the automated in situ hybridization using a modified version of a previously published procedure (Myers et al. 1995). The Ventana Medical Systems (Tucson, Ariz.)

TechMate Automated Staining System was used for this procedure. After deparafinization, the tissues received a mild proteinase K treatment. Hybridization was performed with a probe concentration of 0.1 ng/µl, followed by RNAse treatment, and stringency washes at 2× and 0.1× SSC at 54 °C. Incubation with alkaline phosphatase-conjugated anti DIG antibody was followed by a BCIP-NBT chromogen incubation (violet color). The slides were subsequently counterstained with Eosin (pink).

Results

Isolation of human BPI homologues

Three human cDNAs with similarity to the human BPI amino acid sequence were identified in the HYSEQ expressed sequence tag database. This database has been developed through a unique approach for identifying rarely expressed genes (SBH) (Drmanac and Drmanac 1999).

The deduced amino acid sequence of *BPIL1* encodes an ORF of 458 amino acids (Fig. 1). The amino acid similarity to BPI is 43% (Table 1) but it also displays a significant degree of homology to two probable ligand-binding proteins CAC18887) (42%) and RYA3 (accession no. CAC18886) (42%) (Dear et al. 1991; Beamer et al. 1998). *BPIL1* also displayed (46%) homology to *tenp*, a developmentally regulated gene expressed in chicken brains during neurogenesis (accession no. AAC14583) (Yan and Wang 1998). The Kozak nucleotide consensus sequence (ANNATGG) (Kozak 1987) flanking the initia-

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BPI    MRENMARGPCNAPRWVSLMVLVAIGTAVTAAVNPGVVVRISQKGLDYASQQGTAALQKELKRIKIPDYSD--SFKIKHLGKGHYSFYSMDIREFQLPSSQ  98
LBP    -MGALAR---ALP--SILLALLLTSTPEALGANPGLVARITDKGLQYAAQEGLLALQSELLRITLPDFTG--DLRIPHVGRGRYEFHSLNIHSCELLHSA  92
BPIL2  ---MCTK---TIPVLWGCFLLWNLYVSSSQTIYPGIKARITQRALDYGVQAGMKMIEQMLKEKKLPDLSGSESLEFLKVDYVNYNFSNIKISAFSFPNTS  94
PLTP   --------------MALFGALFLALLAGAHAEFPGCKIRVTSKALELVKQEGLRFLEQELETITIPDLRG-------KEGHFYYNISEVKVTELQLTSSE  79
CETP   --------MLAATVLTLALLGNAHACSKGTSHEAGIVCRITKPALLVLNHETAKVIQTAFQRASYPDITG--EKAMMLLGQVKYGLHNIQISHLSIASSQ  90
BPIL3  --------------MLRILCLALCSLLTGTRADPGALLRL---GMDIMNQVQSAMDESHILE-KMAAEAG----KKQPGMKPIKGITNLKVKDVQLPVIT  78
BPIL1  -----------MAWASRLGLLLALLLPVVGASTPGTVVRLNKAALSYVSEIGKAPLQRALQV-TVPHFLD--------WSGEALQPTRIRILNVHVPRLH  80
                               .*   *:   .:    .     :  :   . .    .                    : :    .

BPI    ISMVPNVGLKFSISNANIKISGKWKAQKRFLKMSGNFDLSIEGMSISADLKLGSNP--TSGKPTITCSSCSSHINSVHVHISK-SKVGWLIQLFHKKIES  195
LBP    LRPVPGQGLSLSISDSSIRVQGRWKVRKSFFKLQGSFDVSVKGISISVNLLLGSE---SSGRPTVTASSCSSDIADVEVDMS--GDLGWLLNLFHNQIES  187
BPIL2  LAFVPGVGIKALTNHGTANISTDWGFESPLFQDTGGADLFLSGVYFTGIIILTRN---DFGHPTLKLQDCYAQLSHAHVSFS--GELSVLYNSFAEPMEK  189
PLTP   LDFQPQQELMLQITNASLGLRFRRQLLYWFFYDGGYINASAEGVSIRTGLELSRD---PAGRMKVSNVSCDASVSRMHAAFG--GTFKKVYDFLSTFITS  174
CETP   VELVEAKSIDVSIQNVSVVFKGTLKYGYTTAWWLGIDQSIDFEIDSAIDLQINTQLTCDSGRVRTDAPDCYLSFHKLLLHLQGEREPGWIKQLFTNFISF  190
BPIL3  LNFVPGVGIFQCVSTG-MTVTG-----KSFMG--GNMEIIVALNITATNRLLRDE---ETGLPVFKSEGCEVILVNVKTNLP----SNMLPKMVNKFLDS  163
BPIL1  LKFIAGFGVRLLAAAN---------FTFKVFRAPEPLELTLP-VELLADTRVTQS---SIRTPVVSISACSLFSCHANEFDG----SNSTSHALLVLVQK  163
       :     :                        :                  :  .                 *                 . .  :

BPI    ALRNKMNSQVCEKVTNSVSSKLQPYFQTLPVMTKIDSVAGINYGLVAPPATTAETLDVQMKGEFYSENH--HNPPPFAP-PVMEFPAAHDRMVYLGLSDY  292
LBP    KFQKVLESRICEMIQKSVSSDLQPYLQTLPVTTEIDSFADIDYSLVEAPRATAQMLEVMFKGEIFHRNH--RSPVTLLA-AVMSLPEEHNKMVYFAISDY  284
BPIL2  PILKNLNEMLCPIIASEVK-ALNANLSTLEVLTKIDNYTLLDYSLISSPEITENYLDLNLKGVFYPLEN--LTDPPFSP-VPFVLPERSNSMLYIGIAEY  285
PLTP   GMRFLLNQQICPVLYHAGTVLLNSLLDTVPVRSSVDELVGIDYSLMKDPVASTSNLDMDFRGAFFPLTE--KNWSLPNR-AVEPQLQEEERMVYVAFSEF  271
CETP   TLKLVLKGQICKEINVISN-IMADFVQTRAASILSDGDIGVDISLTGDPVITASYLESHHKGHFIYKN---VSEDLPLP-TFSPTLLGDSRMLYFWFSER  285
BPIL3  TLHKVLPGLMCPAIDAVLV-YVNRKWTNLSDPMPVGQMGTVKYVLMSAPATTASYIQLDFSPVVQQQKG--KTIKLADAGEALTFPEGYAKGSSQLLLPA  260
BPIL1  HIKAVLSNKLCLSISNLVQ-GVNVHLGTLIGLNPVGPESQIRYSMVSVPTVTSDYISLEVNAVLFLLGKPIILPTDATPFVLPRHVGTEGSMATVGLSQQ  262
        :   :   :*  :         :        .         .     :    :      *  :  . :.        .                 :

BPI    FFNTAGLVYQEAGVLKMTLRDDMIPKESKFRLTTKFFGTFLPEVAKKFPN-MKIQIHVSASTPPHLSVQPTGLTFYPAVDVQAFAV-LPNSSLASLFLIG  390
LBP    VFNTASLVYHEEGYLNFSITDDMIPPDSNIRLTTKSFRPFVPRLARLYPN-MNLELQGSVPSAPLLNFSPGNLSVDPYMEIDAFVL-LPSSSKEPVFRLS  382
BPIL2  FFKSASFAHFTAGVFNVTLSTEEIS--NHFVQNSQGLGNVLSRIAEIYILSQPFMVRIMATEPPIINLQPGNFTLDIPASIMMLTQ-PKNSTVETIVSMD  382
PLTP   FFDSAMESYFRAGALQLLLVGDKVPHDLDMLLRATYFGSIVLLSPAVIDS--PLKLELRVLAPPRCTIKPSGTTISVTASVTIALV-PPDQPEVQLSSMT  368
CETP   VFHSLAKVAFQDGRLMLSLMGDEFK----AVLETWGFNTNQEIFQELSAA--------SPARPKSPSTASRCPRSPAKTRESWSI-LQ----------  360
BPIL3  TFLSAELALLQKS-FHVNIQDTMIG-ELPPQTTKTLARFIPEVAVAYPKSKPLTTQIKIKKPPKVTMKTGKSLLHLHSTLEMFAARWRSKAPMSLFLLE  357
BPIL1  LFDSALLLLQKAGALNLDITGQLRS--DDNLLNTSALGRLIPEVARQFPEPMPVVLKVRLGATPVAMLHTNNATLRLQPFVEVIAT-ASNSAFQSLFSLD  359
        * :          . : . :              :  :                       *       . .

BPI    MHTTGSMEVSAESNRLVGELKLDRLL-LELKHSNIGPFPVELLQDIMNYIVPILVLPRVNEKLQKGFPLPTPARVQLYNVVLQPHQNFLLFGADVVYK--  487
LBP    VATNVSATLTFNTSKITGFLKPGKVK-VELKESKVGLFNAELLEALLNYYILNTFYPKFNDKLAEGFPLPLLKRVQLYDLGLQIHKDFLFLGANVQYMRV  481
BPIL2  FVASTSVGLVILGQRLVCSLSLNRFR-LALPESNRSNIEVLRFENILSSILHFGVLPLANAKLQQGFPLSNPHKFL=VNSDIEVLEGFLLISTDLKYETS  481
PLTP   MDARLSAKMALRGKALRTQLDLRRFR-IYSNHSALESLALIPLQAPLKTMLQIGVMPMLNERTWRGVQIPLPEGIN=VHEVVTNHAGFLTIGADLHFAKG  467
CETP   ----------------------------------------------------------------------------------------------------  360
BPIL3  VHFNLKVQYSVHENQLQMATSLDRLLSLSRKSSSIGNFNERELTGFITSYLEEAYIPVVNDVLQVGLPLPDFLAMNYNIAELDIVENALMLDLKLG----  453
BPIL1  VVVNLRLQLSVSKVKLQGTTSVLGDVQLTVASSNVGFIDTDQVRTLMGTVFEKPLLDHLNALLAMGIALPGVVNLHYVAPEIFVYEGYVVISSGLFYQS-  458

BPI    --------------------------  487
LBP    --------------------------  481
BPIL2  SKQQPSFHVWEGLNLISRQWRGKSAP  507
PLTP   LREVIEKNRPADVRASTAPT?STAAV  493
CETP   --------------------------  360
BPIL3  --------------------------  453
BPIL1  --------------------------  458
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Fig. 1 Alignment of amino acid sequences for human members of the LT/LBP family. Gaps were manually introduced to optimize alignments. Alignments were computed using the Clustal W (1.74) program (Myers and Miller 1988). *Asterisks* indicate identical amino acid residues, *colons* indicate conserved amino acid residues and *dots* indicate residues with weakly-related properties. Conserved cysteine residues have been *boxed*. Accession numbers in the public database for proteins used in this figure are: BPI (P17213), LBP (P18428), CETP (A26941), PLTP (A53533), BPIL1 (AF465765), BPIL2 (AF465766) and BPIL3 (AF465767)

Table 1 Sequence similarity and identity. Initial numbers represent percent similarity between the indicated LT/LBP protein family members, while the numbers in parentheses represent percent identity between family members as determined by using the ALIGN pairwise program (Myers and Miller 1988)

	BPI	LBP	CETP	PLTP	BPIL1	BPIL2	BPIL3
BPI	–	65 (44)	44 (25)	48 (27)	43 (21)	49 (28)	44 (24)
LBP	65 (44)	–	46 (25)	43 (24)	44 (24)	45 (25)	41 (22)
CETP	44 (25)	46 (25)	–	44 (23)	39 (19)	42 (25)	39 (21)
PLTP	48 (27)	43 (24)	44 (23)	–	43 (21)	46 (25)	41 (18)
BPIL1	43 (21)	44 (24)	39 (19)	43 (21)	–	45 (24)	46 (25)
BPIL2	49 (28)	45 (25)	42 (25)	46 (25)	45 (24)	–	42 (22)
BPIL3	44 (24)	41 (22)	39 (21)	41 (18)	46 (25)	42 (22)	–

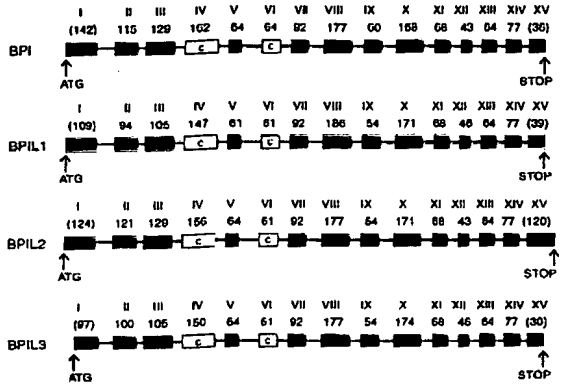

Fig. 2 Gene organization of *BPI*, *BPIL1*, *BPIL2* and *BPIL3*. Exons (*Roman numbers*) and their nucleotide lengths (*Arabic numbers*) are indicated. Introns are not drawn to scale. The locations of the first ATG and the stop TGA codons are shown. Conserved cysteine residues are shown within exons 4 and 6. *Bracketed numbers* reflect the exon sequences corresponding to the open reading frame

tion codon is well conserved and was preceded by an in-frame termination, 22 codons upstream from the putative initiation site. There are four potential N-glycosylation sites [Asn-X-(Ser)/(Thr)] in the predicted sequence. Recently, a full-length cDNA from a human liver HepG2 cDNA library was found to be identical to *BPIL1* (accession no. AK027068). Another human EST (480 nt) with identical sequence to *BPIL1* was found to be downregulated in larynx carcinoma (accession no. AJ403124) (Frohme et al. 2000). Additionally, several mouse cDNAs isolated from a mouse adult male tongue cDNA library appear to be orthologs of *BPIL1* (accession nos. AK010157, AK009754, AK009619 and AK009346). The genomic organization of *BPIL1* was determined by aligning the cDNA sequence to the genomic sequence found in GenBank (accession no. AL121756) (Fig. 2). Protein secondary structure prediction using the computer server SignalP (http://genome.cbs.ctu.dk/services/SignalP) predicted the location of a signal peptide cleavage site immediately after Ala_{20}, indicating that BPIL1 may be secreted.

The deduced amino acid sequence of *BPIL2* encodes an ORF of 507 amino acids (Fig. 1). The amino acid similarity to BPI is 49% (Table 1). An in-frame stop codon is found five codons upstream from the putative initiation codon. There are ten potential N-glycosylation sites in the predicted sequence. Protein secondary structure prediction using the computer server SignalP predicted the location of a signal peptide cleavage site immediately after Ser_{23}. The structure of the *BPIL2* gene was determined by aligning the cDNA sequence to the genomic sequence found in GenBank (accession no. AL021937) (Fig. 2). We have identified several splice variants of *BPIL2*. One splice variant is missing exon 4, known to contain the first of two cysteine amino acid residues totally conserved in the LT/LBP family (Fig. 2). A second variant fails to splice exon 14, resulting in premature termination of the ORF, nine amino acid residues into the adjacent intron sequences. Recently, an EST (485 nt) identical to *BPIL2*, from a brain glioblastoma cDNA library was entered into GenBank (accession no. BF245271).

The deduced amino acid sequence of *BPIL3* encodes an ORF of 453 amino acids (Fig. 1). The amino acid similarity to BPI is 44% (Table 1), but it also displays significant amino acid similarity to the rat proteins RY2G5 (52%) and RYA3 (45%). The genomic organization of *BPIL3* was determined by aligning the cDNA sequence to the genomic sequence found in GenBank (accession no. AL121756) (Fig. 2). A splice variant missing exon 12 was identified. This resulted in a frameshift after Asn_{396}, leading to the premature termination of the ORF, three amino acids downstream from the splice site. There are two potential N-glycosylation sites in the predicted sequence. Protein secondary structure prediction by Signal P predicted the location of a signal peptide cleavage site immediately after Ala_{18}.

The amino acid identity among members of the LT/LBP family ranges from 18% to 44% (Table 1). The highest degree of identity was found among the LPS binding proteins BPI and LBP. This similarity at the functional level is also reflected in the phylogenetic analysis (Fig. 3).

Structural modeling of the BPIL proteins

The three-dimensional structural models of the BPIL proteins were predicted based on a search of 4,250 nonredundant Protein Data Bank structures. The known crystal structure of BPI (Beamer et al. 1997) was the

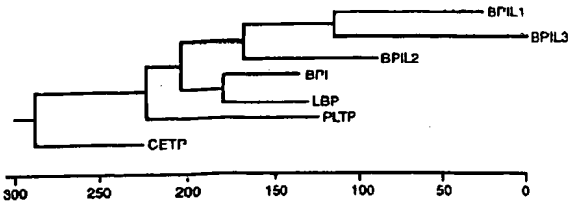

Fig. 3 Phylogenetic analysis of the LT/LBP family. The length of each pair of branches represented the distance between sequence pairs. The *scale* beneath the dendogram indicates the distances between sequences. Dendogram prepared using Clustal W and MegAlign programs (Dnastar, Madison, Wis.).

best-fit structure, which was used as a template to model the BPIL sequences (Fig. 4). These models predict the characteristic boomerang-shape structure, with two similar barrel regions and two hydrophobic potential binding pockets found in the other members of LT/LBP family. The BPIL amino acid sequences also have conserved cysteine residues that are involved in disulfide bond formation between the final strand of the N-terminal beta sheet and the long alpha helix (Cys_{137}-Cys_{174} for BPIL1, Cys_{161}-Cys_{200} for BPIL2, and Cys_{137}-Cys_{174} for BPIL3). Interestingly, the structural regions of the BPIL molecules that correspond to the three previously determined antimicrobial sites of BPI (amino acids 17–45, 82–108, and 142–169) (Little et al. 1994), have fewer positively charged residues compared with *BPI*. In total, these three regions carry the following predicted net charges for the four structures: BPI (+13 charges); BPIL1 (–1 charge); BPIL2 (–6 charges); BPIL3 (+3 charges).

Chromosomal location and genomic structures of BPIL genes

The *BPIL* genes were mapped in vitro using the NIGMS human/rodent somatic cell hybrid mapping panel 2 and the Stanford G3 Human/Hamster Radiation Hybrid panel (Materials and methods). *BPIL1* mapped to Chromosome 20 and to a region close (28 cRs) to marker SHGC-15969 with a LOD score of 8.44. *BPIL3* was mapped to Chr 20 and a region proximal (47 cRs) to the marker SHGC-7112 with a LOD score of 4.44. Markers SHGC-15969 and SHGC-7112 are also in close proximity to the *BPI* locus (SHGC-12397, 20q11-q12), indicating that the *BPIL3*, *BPIL1* and *BPI* genes are close to each other. The proximity of these genes was recently confirmed in silico by the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). *BPIL1* and *BPIL3* (also known as LOC128859 on Map Viewer) were located to the contig NT_028392 while *BPI* was located to the adjacent contig NT_011392. The physical distance between *BPIL3* and *BPIL1* was measured to be 8 kb. The distance between *BPIL3* and *BPI* was approximately 5.0 Mb. *BPIL2* was mapped to Chr 22 in close proximity to the SHGC-10497 marker with a LOD score of 12.33 at a distance of 0 cRs. SHGC-10497 is 15 cRs from the *SLC5A1* locus on 22q12 (Turk et al. 1993). *BPIL2* was identified on the NCBI Map viewer as LOC129103 in contig NT_011520 and it was 305 kb apart from *SLC5A1*.

The ORFs of the *BPIL* genes are distributed over 15 exons and their exon/intron organization is similar to that of *BPI* (Fig. 2).

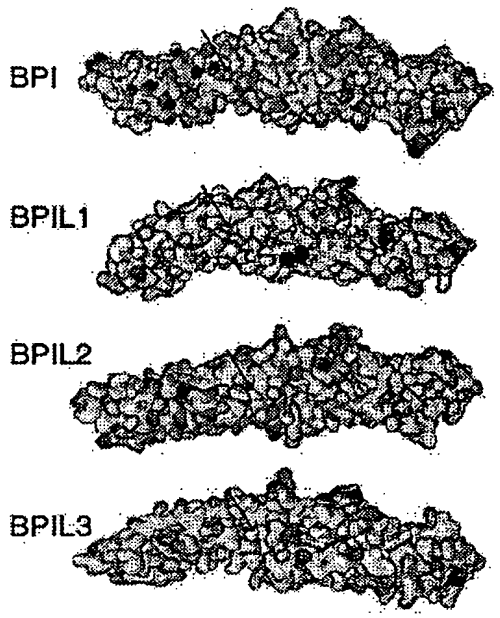

Fig. 4 Surface plots of the crystal structure of BPI and the 3-dimensional models of BPIL1, BPIL2, and BPIL3. Basic (positively-charged) residues are *blue*, while acidic (negatively-charged) residues are *red*. The view is oriented so that the N-terminal barrel is on the *left*. The region containing the phospholipid N-terminal barrel binding pocket is indicated by the *arrows*

Expression of BPILs in different tissues

Since *BPI* expression is restricted to neutrophils and eosinophils, the *BPIL* genes were screened using a multiple tissue cDNA panel from immune related tissues (Materials and methods). PCR amplification of cDNAs from 7 normalized tissues revealed that *BPIL3* was expressed only in tonsil (Fig. 5). *BPIL1* expression was detected in tonsil along with a very low level detected in fetal liver. *BPIL2* expression was not detected in this panel of tissues. Interestingly, the expression of *BPI*, which was found to occur preferentially in the bone marrow, did not overlap with that of the *BPIL* genes (Fig. 5). Expression of *BPIL1*, *BPIL2* and *BPIL3* was not detected in other cDNA panels (see Materials and methods for a list of tissues tested) (data not shown). SBH expression data found *BPI* abundantly expressed in a leukocyte cDNA library, consistent with its expression in neutrophils and eosinophils, while *BPIL3* and *BPIL1* were found at very low levels in a trachea cDNA library and *BPIL2* was found in a fetal skin library.

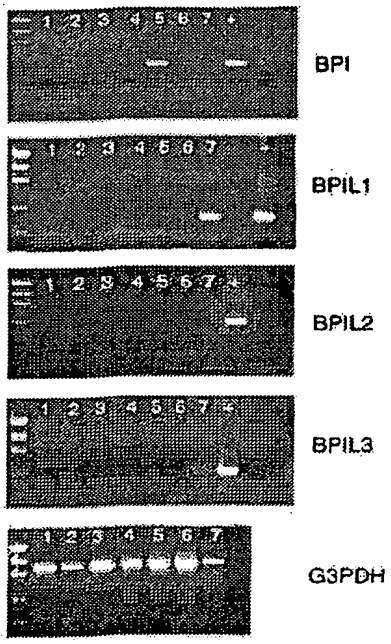

Fig. 5 Expression analysis of *BPIL1*, *BPIL2* and *BPIL3*. Gene expression was assessed by semi-quantitative PCR amplification using gene-specific primers and first strand cDNAs from a panel of human immune tissues (Materials and methods). Copy DNAs were from: *1* spleen, *2* lymph node, *3* thymus, *4* PBL, *5* Bone marrow, *6* fetal liver and *7* tonsil

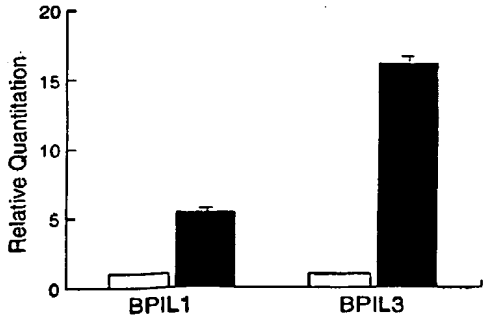

Fig. 6 Quantitative expression of *BPIL1* and *BPIL3* in normal vs hypertrophic tonsil. The expression was determined by real-time fluorescent RT-PCR in normal (*white bars*) and hypertrophic (*black bars*) tonsils. Amplifications of first strand cDNA were performed with gene-specific primers, and expression levels were normalized to those of cyclophilin 40

The expression of *BPIL3* and *BPIL1* in tonsil was studied in more detail using a real-time PCR approach. Normal and hypertrophic tonsil first strand cDNA libraries were assayed with primers specific for *BPIL3*, *BPIL1*, as well as the housekeeping genes cyclophilin 40 (*CYPD*) and the ribosomal protein gene *RPS21*. We found that the expression of *BPIL1* (five-fold) and *BPIL3* (16-fold) was significantly up-regulated in the hypertrophic samples while the expression of cyclophilin

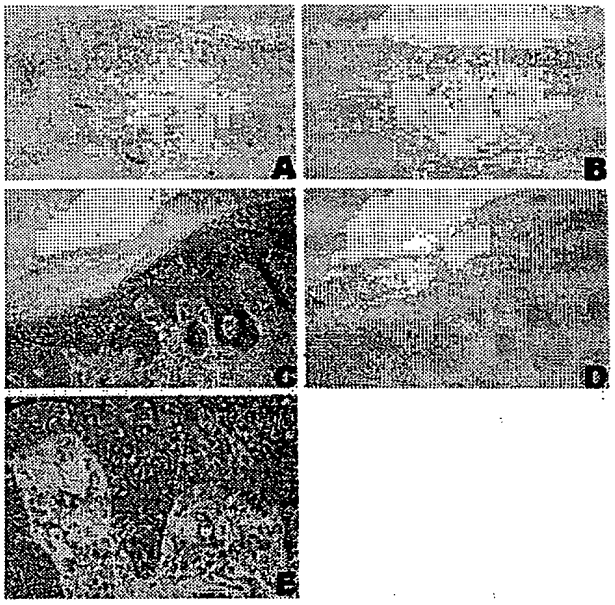

Fig. 7a–e In situ hybridization of DIG-labeled *BPIL2* riboprobes to human skin tissue sections. **a** 100× magnification of normal forearm skin hybridized with antisense probe. **b** 100× fetal skin hybridized with antisense probe. **c** 100× psoriasis forearm skin hybridized with antisense probe. **d** 100× psoriasis forearm skin hybridized with sense probe. **e** 200× High-power view showing preferential staining of the basal cells of the epidermis in the skin specimen with psoriasis

40 remained constant (Fig. 6). Similar results were obtained when the samples were normalized to the expression of the housekeeping gene *RPS21* (data not shown).

The *BPIL2* cDNA was initially identified as a transcript from a human fetal skin library. To localize the expression in skin, in situ hybridization analysis of *BPIL2* expression was performed (Fig. 7). Skin samples from fetal, adult normal, and psoriasis specimens were analyzed. A sequence-specific probe for *BPIL2* failed to detect expression in fetal and adult skin. However, binding of the probe was detected prominently in the basal layer of the epidermis from inflammatory skin of psoriasis specimens.

Discussion

We have identified three novel human cDNAs that encode proteins sharing structural and sequence homologies with the Lipid transport/LPS binding protein (LT/LBP) family members (*BPI*, *LBP*, *PLTP* and *CETP*). The amino acid sequences for the three BPIL proteins showed regions of similarity with the LT/LBP members extending over the full length of the proteins. The most salient feature is the conservation of two cysteine residues, known to anchor the amino terminal domain of the BPI protein (Beamer et al. 1997). These two cysteines are critical for the function of BPI (Horwitz et al. 1996)

and are conserved in all members of the LT/LBP family. *BPIL1*, *BPIL2* and *BPIL3* also display amino acid similarity to two rat proteins (RY2G5 and RY3) thought to be relatives of the LT/LBP family (Beamer et al. 1998).

Structural modeling of the three new BPIL proteins utilizing homology modeling (Sali and Overington 1994) or a modified threading method (Burstein et al. 2000) reveals that the amino acid sequences and properties from all three proteins make it possible for them to share a conformation similar to that of BPI. Like the other members of the LT/LBP family, all three of these BPIL molecules are predicted to include two hydrophobic pockets, which may transport small hydrophobic ligands. Although there is structural similarity in the backbone of these molecules and the crystal structure of BPI, significant differences exist in the amino acid side-chains, resulting in differential charge distribution at the surfaces. Of note is the net charge in the N-terminal regions of BPI that have been implicated in LPS-binding, LPS-neutralization, and bactericidal activity (Little et al. 1994). Interestingly, like other members of this family of proteins that lack bactericidal activity, CETP and PLTP (which have neutral and +4 charges, respectively), the BPIL proteins have negative or only weakly positive net charges in these regions as compared to BPI.

BPIL1 and *BPIL3* were mapped to a region at or near 20q11-q12, resulting in five closely related genes, including *BPI*, *LBP* and *PLTP*, located in this region. The other two members, *CETP* and *BPIL2* are located on Chrs 16 and 22, respectively. Analysis of the *BPIL1*, *BPL2* and *BPIL3* exon/intron boundaries revealed that the ORFs were distributed among 15 exons (Fig. 2). *BPI* and *PLTP* also have 15 exons, while *LBP* and *CETP* have 14 and 16 exons, respectively (Hubacek et al. 1997). The exon size is also highly conserved, suggesting a common origin for all these genes, with different degrees of divergent evolution.

Each of the three new members of the LT/LBP gene family showed low levels of expression in normal tissues. Though *BPIL2* was originally identified as a rarely expressed gene in fetal skin, expression could not be detected in any of the cDNA panels used in our studies (Fig. 5 and Materials and methods). In situ hybridization likewise failed to detect expression in fetal and normal adult skin tissues. However, expression of the gene was detected within the basal cell layers of the epidermis in skin samples from psoriatic tissue. The expression of the *BPIL1* and *BPIL3* genes was detected in tonsils (Fig. 5). We confirmed and compared the expression in normal and hypertrophic tonsil samples using a quantitative real-time PCR approach (Fig. 6). Interestingly, both genes were upregulated in the hypertrophic tonsil, suggesting that they may play a role in disease progression.

The sequence similarity to the LT/LBP family suggests that the BPIL proteins likely perform similar fundamental functions in vivo. Their restricted pattern of expression, in particular their overexpression in inflamed disease tissues, suggests that these genes may play a role in innate immunological functions. Possible roles for these proteins include binding small hydrophobic ligands that play roles in inflammation, host defense or pain, such as prostaglandins, leukotrienes and lipoamino acids (Huang et al. 2001; Chenchik et al. 1996). Alternatively, small molecules shed by invading microorganisms could also serve as ligands for these proteins. Such interactions could in turn trigger an immune response as in the case of the LBP protein and its interaction with bacterial LPS.

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